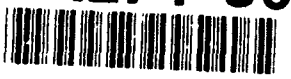


1 June 1993 Reprint

Isolation and Characterization of Two Campylobacter Glycine-Extracted Proteins That Bind to HeLa Cell Membranes

Army Project Order 90PP0820

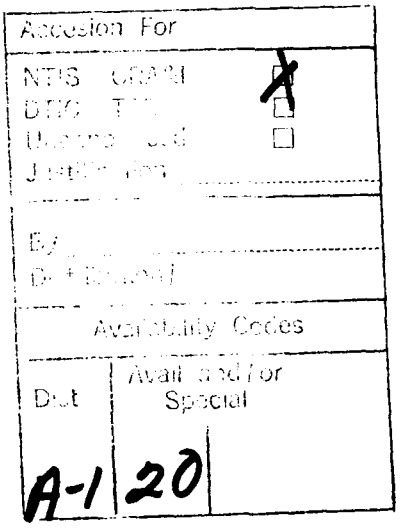
Michele Kervella, Jean-Marie Pages, Zhiheng Pei, Ghislaine Grollier, Martin J. Blaser, and Jean-Louis Fauchere

Veterans Administration Medical Center
Research Service
1310 24th Avenue South
Nashville, Tennessee 37212-2637

U.S. Army Medical Research & Development Command
Fort Detrick
Frederick, Maryland 21702-5012

Title of Project Order: Studies of the Outer Membrane Proteins Campylobacter Jejuni for Vaccine Development

Approved for public release; distribution unlimited



Campylobacter, Vaccines, Biotechnology, ID, Diseases
RADI, Lab Animals, Volunteers

Unclassified Unclassified Unclassified Unlimited

INFECTION AND IMMUNITY, Aug. 1993, p. 3440–3448
0019-9567/93/083440-09$02.00/0

Vol. 61, No. 8

Isolation and Characterization of Two *Campylobacter* Glycine-Extracted Proteins That Bind to HeLa Cell Membranes

MICHÈLE KERVELLA,[1] JEAN-MARIE PAGÈS,[2] ZHIHENG PEI,[3] GHISLAINE GROLLIER,[4] MARTIN J. BLASER,[3] AND JEAN-LOUIS FAUCHÈRE[4]*

Laboratoire de Bactériologie, Faculté de Médecine Necker-Enfants Malades, 75730 Paris Cédex 15,[1] Centre de Biochimie et de Biologie Moléculaire, Centre National de la Recherche Scientifique, BP 71, 13402 Marseille Cédex 9,[2] and Laboratoire de Microbiologie A, CHU la Miletrie, 350 avenue Jacques Coeur, BP 577, 86021 Poitiers,[4] France, and Division of Infectious Diseases, Department of Medicine and Department of Microbiology and Immunology, Vanderbilt University School of Medicine and Department of Veterans Affairs Medical Center, Nashville, Tennessee 37232-2605[3]

Received 21 January 1993/Accepted 1 June 1993

Two immunogenic proteins of 27 (CBF1) and 29 (CBF2) kDa from enteropathogenic *Campylobacter* species appear to bind to mammalian cells. We purified these two proteins from a pathogenic and adherent *Campylobacter jejuni* strain to homogeneity by using acid extraction, preparative gel electrophoresis, and electroelution. Polyclonal rabbit antisera to these proteins were prepared. Immunologic studies indicate that CBF1 corresponds to the PEB1 and CBF2 corresponds to the PEB4 described by Pei et al. (Z. Pei, R. T. Ellison, and M. Blaser, J. Biol. Chem. 226:16363–16369, 1991). Immunogold labeling of a *C. jejuni* adherent strain with anti-CBF1, anti-CBF2, and anti-PEB1 suggested that CBF1 (PEB1) is surface exposed while CBF2 (PEB4) is not. Analysis of whole-cell extracts from 14 strains by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with 7 M urea and immunoblotting with antisera to CBF1 and CBF2 suggests that CBF proteins from adherent and nonadherent strains are different. Use of purified proteins in a microassay of adherence to cellular membranes indicated that CBF1 was much more adherent than CBF2. Adherence of *C. jejuni* to viable HeLa cells was markedly reduced with the antiserum to CBF1, whereas the CBF2 antiserum was a poor inhibitor. Purified CBF1 competitively inhibited adherence of whole bacteria to HeLa cells, whereas purified CBF2 was no better a competitor than bovine serum albumin. Adherence of CBF2 was markedly reduced in the presence of Tween 20 or SDS, whereas adherence of CBF1 was reduced only by SDS. We conclude that (i) CBF1 (PEB1) is surface exposed and may be the key protein for *C. jejuni* adhesion and (ii) CBF2 (PEB4) may be complexed with CBF1 and may passively coadhere with CBF1 under certain experimental conditions. Adherent and nonadherent strains contain different isotypes of these two proteins which could be useful markers of *C. jejuni* adhesion.

Campylobacter jejuni and *C. coli* are major causes of enteritis in humans throughout the world (12, 30); however, little is known about the virulence factors that contribute to the pathogenesis of *Campylobacter* enteritis. Clinical and experimental evidence indicating adhesion to or invasion of human epithelial cells by pathogenic *Campylobacter* strains suggests that such phenomena are important events in the pathogenesis of *Campylobacter* infection (4, 14, 18, 25, 31) and in any case, adhesion may be considered the first step of bacterium-intestinal cell interaction. Several investigators have reported adherence properties of *Campylobacter* species either in vitro (4, 13, 25) or in vivo (14, 31). In previous work, we showed that in vitro adherence correlated with virulence in humans (13). In other studies, invasive properties of *C. jejuni* have been shown (20). Thus, molecular studies of *Campylobacter* adherence are of interest to help understand the pathophysiology of *Campylobacter* enteritis.

As for other enteropathogenic organisms, adhesion may involve bacterial surface-exposed structures (colonization or adhesion factors) able to recognize and bind to specific receptors of intestinal mucosal cells. We previously reported that a group of proteins including two major cell-binding fractions (CBFs) of approximately 27 (CBF1) and 29 (CBF2) kDa present in adherent *C. jejuni* strains specifically adhere to HeLa cells (11). Antiserum raised against this group of proteins inhibited the binding of whole *C. jejuni* cells to epithelial cells better than does an antiserum raised against a nonadherent surface protein. Moreover, CBFs were among the most immunogenic bacterial constituents for infected patients (11, 18). Following this initial work, two proteins with migration on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of 28 to 32 kDa were identified by de Melo and Pechère as possible adhesins on the basis of similar results (5). Simultaneously, Konkel et al. studied a surface protein of 38 to 42 kDa which they thought to be associated with penetration of *C. jejuni* into epithelial cells (19).

Thus, a number of investigators believe that *Campylobacter* outer membrane proteins ranging between 25 and 42 kDa play a role in bacterium-cell interactions but none of these investigators purified these proteins to study their properties when isolated from bacterial cells. On the other hand, other researchers (6, 27) have purified and characterized several *Campylobacter* proteins in this molecular weight range but without any reference to their adherence functions.

In this work, we tried to bring together these two approaches by purifying CBF1 and CBF2. We demonstrated

* Corresponding author.

TABLE 1. Characteristics of the strains studied

Strain	AI[a]	Presence of:		
		Diarrhea	Fever	Blood in feces
C. jejuni 85AC	7.1	+	−	−
C. coli 79K	6.8	+	+	+
C. coli 87C	6.6	+	+	+
C. jejuni 85H	6.5	+	+	+
C. jejuni 87R	5.6	+	−	−
C. jejuni 86AC	5.5	+	+	+
C. jejuni 85V	3.8	+	+	−
C. jejuni 85AE	1.0	−	−	−
C. jejuni 85R	0.8	−	−	−
C. coli 85AR	0.3	−	−	−
C. jejuni 85D	0.2	+	+	−
C. jejuni 85AQ	0.1	−	+	−
C. jejuni 79AH	0.1	−	−	−
C. coli 85Y	0.0	−	−	−

[a] Weighted mean of number of bacteria per cell in the association test (13).

that these two proteins are identical to previously recognized *C. jejuni* antigens (27), we localized these proteins on the bacterial cell, and we studied their distribution among adherent and nonadherent campylobacters. We then studied the adherence properties of these two isolated proteins by comparing their binding to cellular membranes with that of other purified *Campylobacter* proteins. Finally, we studied inhibition of whole bacterial cell adhesion by these proteins or by their specific antisera.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Fourteen strains of *C. jejuni* and *C. coli* (Table 1) were isolated from human feces: seven (79K, 87C, 85H, 86AC, 85AC, 85V, and 87R) were highly adherent, and seven (85AR, 85AQ, 79AH, 85Y, 85D, 85AE, and 85R) were poorly adherent or nonadherent on the basis of the adherence assay described previously (13). Stock cultures, kept at −80°C, were rapidly thawed and grown for 24 h on Columbia agar (Difco, Detroit, Mich.) at 37°C in a humid incubator under microaerobic conditions.

Glycine extraction of bacterial proteins. Extraction of proteins from *Campylobacter* species was performed by the method of McCoy et al. (24) as modified by Logan and Trust (22). Cultures in 50 Roux flasks with Columbia agar were harvested in distilled water. The surface protein antigens were separated from bacterial cells by stirring the cells in 0.2 M glycine buffer (pH 2.2) for 15 min at room temperature. Cells were centrifuged at 10,000 × *g* for 15 min. The supernatant was neutralized with NaOH, dialyzed against distilled water, and lyophilized. The protein contents of the glycine extracts were determined by the bicinchoninic acid method (28).

Electrophoresis. The method of Laemmli (21) was used for standard SDS-PAGE. Glycine extract preparations were stacked in a 5% gel and separated in a 12% acrylamide gel. In some experiments, bacterial cell pellets also were analyzed. The pellets were solubilized in 160 mM Tris-HCl (pH 8.8)–0.8 M sucrose–4 M EDTA–3.6% SDS–60 mM dithiothreitol–1% 2-mercaptoethanol as previously described (26). The migration of these samples in SDS–11% acrylamide gels containing either 7 M or no urea was determined as previously described (26). After electrophoresis at 80 mA for 4 h, proteins were resolved with either Coomassie blue or by the very sensitive double-staining technique of Dzandu et al. (8), which is known to detect both lipopolysaccharide and proteins. As needed, the separated proteins were immunoblotted (3) by incubation with appropriate sera and then with peroxidase- or alkaline phosphatase-conjugated second antibodies and bands were resolved as previously described (11).

Protein purification. Several proteins (CBF1, CBF2, and flagellin [FLA]) from glycine extracts of *C. jejuni* 85H were purified to homogeneity. Proteins were separated by one-dimensional preparative SDS-PAGE (gel dimensions, 180 by 140 by 13 mm) under the standard experimental conditions described above. The excised gel slices containing the protein bands were cut into fragments and electroeluted as previously described (29). Gel pieces were incubated in elution buffer (25 mM Tris, 192 mM glycine) containing 0.025% SDS for 30 min and electroeluted in the buffer overnight (100 V, room temperature) in a Biotrap apparatus (Schleicher & Schüll, Cera Labo, France). Following electroelution, protein eluates were electrodialyzed in Tris-glycine buffer (25 mM Tris, 192 mM glycine) for 4 h (200 V, room temperature) to remove SDS, protein concentrations were determined (28), and samples were subjected to analytical SDS–11% PAGE to assay for protein purification to homogeneity. Proteins PEB1, PEB2, PEB3, and PEB4 were purified to homogeneity from glycine extracts of *C. jejuni* 81-176 as previously described (27).

Sera. Hyperimmune rabbit antisera to whole bacterial cells were prepared as previously described (11). Rabbit antiserum to whole cells of *C. jejuni* 85H has been previously shown to recognize glycine extracts of strains 85H and 79AH. Antiserum to PEB1 from *C. jejuni* 81-176 was prepared as previously described (27).

Rabbit antisera to isolated proteins CBF1 and CBF2 and a 23-kDa protein (P23) were prepared essentially as previously described (11). Briefly, 30 μg of the protein in acrylamide obtained by SDS-PAGE separation of a glycine extract was homogenized with saline and Freund's complete adjuvant and used to immunize a rabbit by subcutaneous injections. Booster doses were given 2 and 4 weeks later. Sera were collected during week 6. The rabbit sera were adjusted to the same titer as previously described (11). Preimmune serum was obtained from each rabbit.

The specificity of sera to the isolated proteins was checked by both enzyme-linked immunosorbent assay (ELISA) and Western blot (immunoblot). For ELISA, 1 μg of glycine extract or purified antigens was used to coat each microtiter plate well. The sera studied were then added at various dilutions, and after 1 h at 37°C, the wells were washed and the bound antibodies were quantitated by using peroxidase-conjugated goat anti-rabbit antibodies. For Western blotting, glycine extracts or purified antigens were resolved by SDS-PAGE and electrotransferred to nitrocellulose and Western blotting was performed.

Culture of HeLa cells. HeLa cells were cultured as previously described (13). Briefly, HeLa cell monolayers on coverslips placed in 24-well plates (Greiner, Paris, France) were maintained in minimum essential medium (MEM) with 10% newborn calf serum (Eurobio, Paris, France). The antibiotics used were streptomycin (50 μg/ml), penicillin (200 U/ml), and amphotericin B (2.5 μg/ml). Cultures were incubated at 37°C in a 5% CO_2 humidified atmosphere to approximately 50% confluency for adhesion experiments.

Adhesion inhibition tests. The ability of antisera or proteins to inhibit adherence of *C. jejuni* 85H to HeLa cells was determined as previously described (11), with some modifi-

cations. Before each assay, cell monolayers were washed three times with MEM (serum and antibiotic free).

For inhibition by an antiserum, bacteria were suspended at 10^9 CFU/ml in MEM containing appropriate dilutions of the serum tested. Serum from an unimmunized rabbit was used as a noninhibiting control. These suspensions were incubated for 1 h at 37°C with shaking, and then bacterial suspensions were washed three times and incubated at 37°C for 1 h with the monolayers at a ratio of 500 to 1,000 CFU per HeLa cell to allow bacterial adhesion to the cells. The cells were then washed 10 times with MEM and fixed with methanol. During such short incubations, bacteria associated with HeLa cells are mainly adherent; internalization requires an additional 3 h of incubation in this system (13). To detect adherent bacteria, coverslips were stained by an immunoassay as described by Evans et al. (9). Cell monolayers with adherent bacteria were incubated with rabbit antiserum raised against whole homologous bacterial cells at a predetermined optimum dilution and then with goat anti-rabbit immunoglobin conjugated with horseradish peroxidase (Prosan, Sebia, France). The substrate was 0.06% diaminobenzidine (Sigma) in 0.05 M Tris-HCl buffer (pH 7.6) and 30% hydrogen peroxide (1/400, vol/vol). As a result of this procedure, bacteria were stained dark brown and cells were then counterstained with carbol fuchsin. One hundred randomly chosen cells were observed by light microscopy, and adhesion of bacteria was expressed as the mean number of adherent bacteria per HeLa cell (association index [AI]). Under these experimental conditions, the microscopic evaluation of adherent bacteria correlates well with the viable count evaluation. The 100% adhesion control was performed by replacing serum with MEM. Each assay was repeated three to six times, and the coefficients of variation (i.e., the standard deviation divided by the mean) for the AI ranged from 3 to 20%.

For the inhibition assay with the purified proteins, HeLa cell monolayers were incubated for 60 min at 37°C with 10, 1, or 0.1 μg of the purified protein or with 10 μg of bovine serum albumin (BSA), used as a noninhibiting control protein. After three washes with MEM, whole bacteria were introduced and the subsequent steps were done as described above. Each assay was run at least twice.

Preparation of HeLa cell membranes. Preparation of HeLa cell membranes was performed as previously described (10). In brief, monolayers from 15 to 20 confluent flasks of HeLa cells were washed twice with isotonic phosphate-buffered saline (PBS, pH 7.4). The cells were lysed at 4°C for 2 h under pressure in 1 mM sodium bicarbonate containing a protease inhibitor (0.1 mM phenylmethylsulfonyl fluoride). The lysate was centrifuged at 27,000 × *g* for 15 min at 4°C, and the pellet containing the organelles and cytoplasmic membrane was resuspended in 5 ml of sterile distilled water (membrane-enriched fraction) and stored at −20°C before use. The protein content of the membrane-enriched fraction was determined by the technique of Smith et al. (28). These membrane-enriched fractions were used as cell receptors in the adherence microassay described below.

Adherence microassay. Adherence of isolated proteins was determined by a previously described microtiter assay (10). Briefly, microtiter plates were coated with the membrane-enriched fraction from HeLa cells and excess binding sites on the polystyrene were blocked with BSA. After washing of the wells, known quantities of the bacterial component were added and plates were incubated for 1 h at 37°C and then washed three times with PBS (pH 7.4) alone or containing either 0.05% (wt/vol) Tween 20 or 0.05% (wt/vol) SDS in

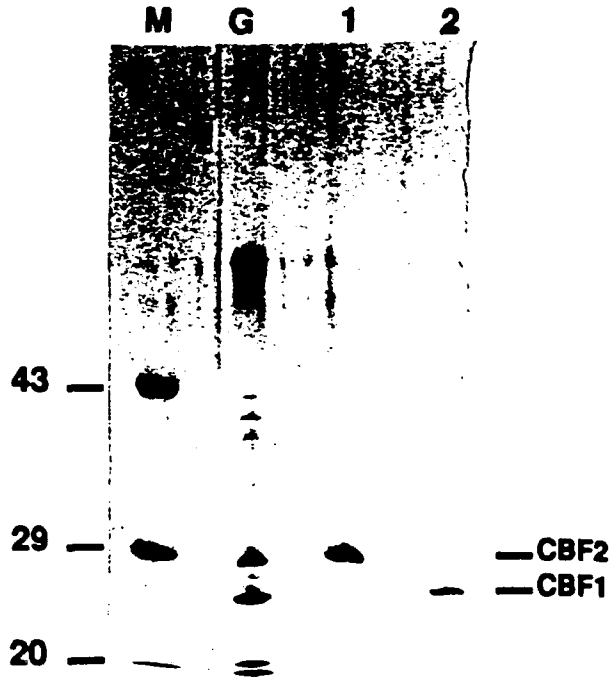


FIG. 1. Purification of proteins CBF1 and CBF2 from *C. jejuni* 85H. Proteins were analyzed by SDS-PAGE (11% acrylamide) and double stained (8). Lanes: M, standard molecular size markers (in kilodaltons on the left); G, glycine extract from 85H; 1, purified CBF2; 2, purified CBF1.

later experiments. The relative amount of material bound was determined by an ELISA using rabbit anti-*C. jejuni* sera and peroxidase-conjugated goat anti-rabbit immunoglobulin as the second antibody. This assay provides an optical density (OD; adherence OD) that represents the adherence of the components studied. When we compared the adherence of two particular components to make sure that each was similarly immunodetected, wells were directly coated with known quantities of the bacterial components studied and the coating material was detected as described above. The resulting ODs (calibration ODs) represented the quantities of bacterial components coating the wells, and the resulting calibration curves for the components studied were found to be superimposable. Adherence values were then normalized with reference to the calibration ODs, permitting direct comparisons of the different components.

Immunogold labelling for electron microscopy. Bacteria (strain 85H) were routinely cultured on Columbia agar for 24 h. Bacterial cells were harvested and suspended in 0.15 M NaCl with 4% formaldehyde. After 15 min at room temperature, they were washed three times in PBS (pH 7.4) with 1% BSA and 0.5% gelatin (PBS-BG). A grid covered with Formvar and carbon-treated (JEOL JEE 4X) film was immersed in the bacterial cell suspension in PBS-BG for 30 min. The grid was then floated for 2 h in the first antiserum diluted at 1/10 in PBS-BG and rinsed three times in PBS-BG. The grid was then floated in PBS-BG containing 10% goat anti-rabbit immunoglobulin G conjugated with 10-nm-diameter colloidal gold particles (Amersham). After 60 min of incubation, the grid was rinsed in PBS-BG, rinsed again in

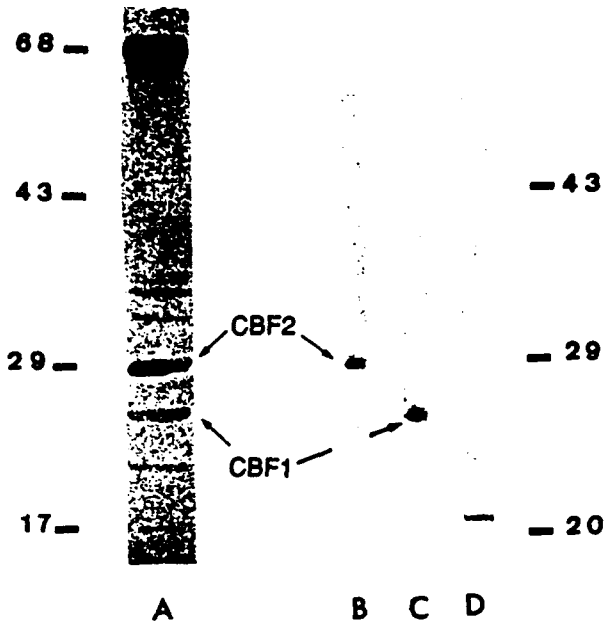


FIG. 2. Recognition of glycine extract from *C. jejuni* 85H by antisera raised against specified antigens. Lanes: A, anti-whole-cell serum; B, anti-CBF2 serum; C, anti-CBF1 serum; D, anti-P23 serum. Each serum had previously been adjusted to the same titer and was used at a dilution of 1:500 for immunoblotting. The numbers are molecular sizes in kilodaltons.

distilled water, and examined in a 100-CX electron microscope (JEOL).

RESULTS

Purification of proteins CBF1 and CBF2 from strain 85H. Because of their capacity to bind to HeLa cells, two proteins of *C. jejuni*, referred to as CBFs, with approximate molecular masses of 27 (CBF1) and 29 (CBF2) kDa were believed to mediate the attachment of *C. jejuni* and *C. coli* to enterocytes (11). CBFs were initially identified in Sarkosyl extracts (11), but the most efficient recovery was obtained with the glycine extraction procedure described by McCoy et al. (24). SDS-PAGE analysis of the glycine extract from adherent *C. jejuni* 85H showed that 65 (flagellin [FLA])-, 29-, 27-, and 23-kDa proteins were the major components (Fig. 1, lane G), similar to previous reports (1, 2, 22, 23). CBF1, CBF2, and FLA, which was used as a nonadherent-protein control, were excised and purified from preparative gels. Elimination of SDS by dialysis should permit renaturation of each protein.

As shown by double-stained SDS-PAGE (Fig. 1, lanes 1 and 2), the purified CBF1 and CBF2 preparations always migrated at the expected locations. No other protein or lipopolysaccharide was detected by the double-staining technique. That each protein was resolved in only a single band indicates that no degradation had occurred during purification.

Specificity of anti-CBF sera. The antisera to CBF1, CBF2, and P23 assayed by Western blot against the homologous glycine extract each recognized only a single band at the expected location (Fig. 2). The anti-CBF sera were assayed by ELISA and Western blot against the previously purified proteins (PEB1 to PEB4) known to have molecular weights in the same range (27). By ELISA, anti-CBF1 recognized PEB1, as well as the homologous glycine-extract, but did not recognize PEB2, PEB3, or PEB4 (Fig. 3A). Anti-CBF2 recognized PEB4, as well as the homologous glycine extract, but did not recognize PEB1, PEB2, or PEB3 (Fig. 3B). These results were confirmed by Western blotting (Fig. 4), which showed that anti-CBF1 recognized PEB1 exactly as did the antiserum raised to PEB1 and showed no recognition of the other proteins. Similarly, anti-CBF2 recognized PEB4 but none of the other proteins. The similarity in reported molecular weights and the antigenic cross-reactivities indicate that CBF1 is PEB1 and CBF2 is PEB4.

Distribution of CBF1 and CBF2 in *C. jejuni* and *C. coli* strains. All strains of *C. jejuni* and *C. coli* contain proteins in the 26- to 30-kDa region, as shown by one- and two-dimensional gel electrophoresis (1, 7, 27). Under the classic experimental conditions of SDS-PAGE, the protein profiles in this region are similar for adherent and nonadherent

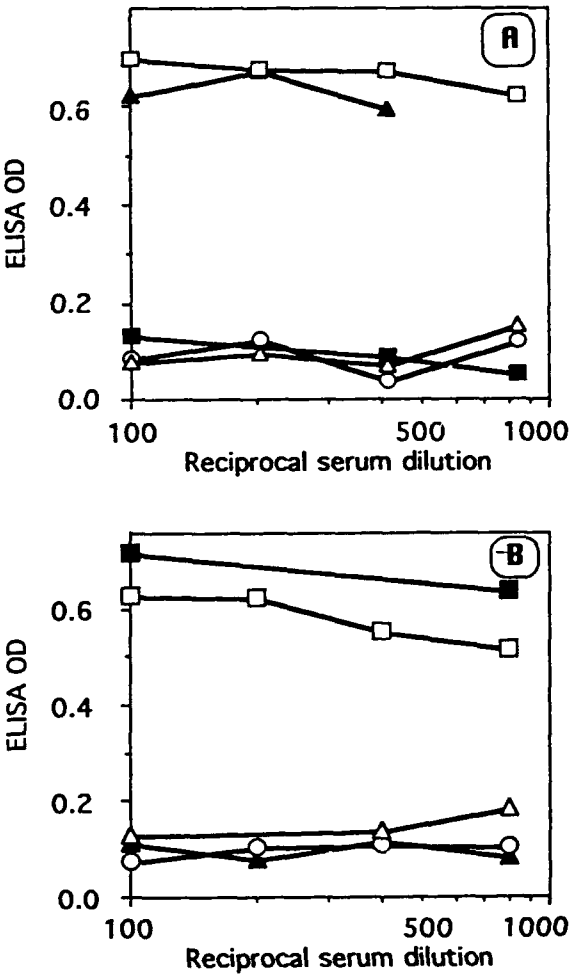


FIG. 3. Recognition of purified *C. jejuni* antigens by antisera to CBF1 (A) and CBF2 (B). Standard antigen concentrations (1 μg) were adsorbed onto microtiter wells, and then serial dilutions of hyperimmune rabbit sera to CBF1 and CBF2 were added. After use of a horseradish peroxidase-conjugated anti-rabbit antibody as the second antibody, OD was determined as previously described (10). The antigens used were purified PEB1 (▲), PEB2 (○), PEB3 (△), PEB4 (■), and total glycine extract (□).

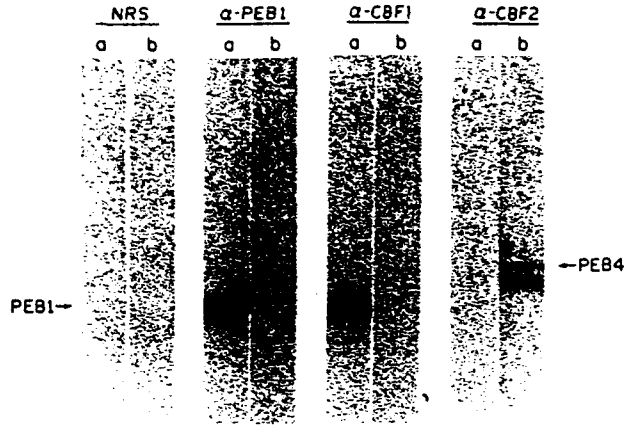


FIG. 4. Immunoblotting of PEB1 and PEB4 with rabbit sera. Purified PEB1 (lanes a) and PEB4 (lanes b) were subjected to SDS-PAGE with 10% acrylamide and transferred to nitrocellulose paper. The sera shown were from an unimmunized rabbit (NRS) or rabbits hyperimmunized with PEB1 (α-PEB1), CBF1 (α-CBF1), or CBF2 (α-CBF2). The conjugate used was alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin. Both of the antisera to PEB1 and CBF1 recognized PEB1, and anti-CBF2 serum recognized PEB4.

strains. To distinguish proteins CBF1 and CBF2 from the two different types of strains, we performed an electrophoretic separation of total cell extracts under more stringent conditions by incorporating 7 M urea into SDS-PAGE. The gels then were immunoblotted with antiserum to CBF1 or CBF2. The migrations of CBF1 and CBF2 were indistinguishable for adherent and nonadherent strains when analyses were performed in the usual 11% acrylamide SDS-PAGE (Fig. 5, upper panels), although the signal intensity varied. For example, no product was recognized by anti-CBF1 serum in the extract of strain 85R and only a weak response was obtained for strain 79AH.

The pattern of immunodetected products was somewhat different when the urea–SDS-PAGE system was used to separate the total cell proteins (Fig. 5, lower panels). First, the reacting material was heterogeneous, especially with anti-CBF1. In the case of nonadherent strains, numerous bands were observed, indicating variation in the antigens recognized. Second, the pattern obtained with nonadherent strains was generally more diffuse than that obtained with adherent strains. The bands were regular (brick shaped) for the adherent strains, while they had a mustache shape or a ladder shape for the other strains. Finally, as expected from the regular SDS-PAGE results, no cross-reacting material was detected with anti-CBF1 for nonadherent strain 85R and little was detected for nonadherent strain 79AH. With antiserum to CBF2, only a slight response to strain 85Y was observed.

Localization of CBFs on the bacterial cell. Immunogold labelling of *C. jejuni* 85H was performed with either anti-CBF1, anti-CBF2, or anti-PEB1 as the first antibody. Antiserum to glycine-extracted proteins between 25 and 45 kDa, including the major outer membrane protein of 45 kDa, was used as a positive control, and preimmune rabbit serum was used as negative control. Anti-CBF1 (Fig. 6A) and anti-PEB1 (Fig. 6D) bound to the surface of the bacterial cell or to the released bacterial material, while anti-CBF2 did not (Fig. 6B). The intensity of labelling was greater with the positive control serum, because this serum recognizes several proteins on the cell surface (Fig. 6E). Preimmune rabbit serum did not bind to the bacterial cell (Fig. 6C). These results suggest that CBF1 (PEB1) is surface exposed whereas CBF2 (PEB4) is inaccessible to the surface.

Binding of purified CBF1 and CBF2 to epithelial cell membranes. A microassay of adherence to immobilized HeLa cell membranes (10) was used to determine whether the purified CBFs possess adherence properties (as identified by previous studies of crude preparations [11]). In this assay, the components remaining adherent on HeLa cell membranes after washing with PBS containing 0.05% Tween 20 were immunodetected with immune serum to whole cells of *C. jejuni*. The adherence of purified CBF1, CBF2, and FLA was compared with that of crude glycine extracts from two different strains, adherent strain 85H and poorly adherent strain 79AH (Fig. 7A). As expected, the glycine extract from strain 79AH did not bind to membranes, which correlates with the low-adherence property of 79AH whole cells. In contrast, the glycine extract from strain 85H was highly adherent, demonstrating that the adherence microassay correlates well with the assay performed on viable HeLa cells (13). The adherence of purified FLA and CBF2 was low,

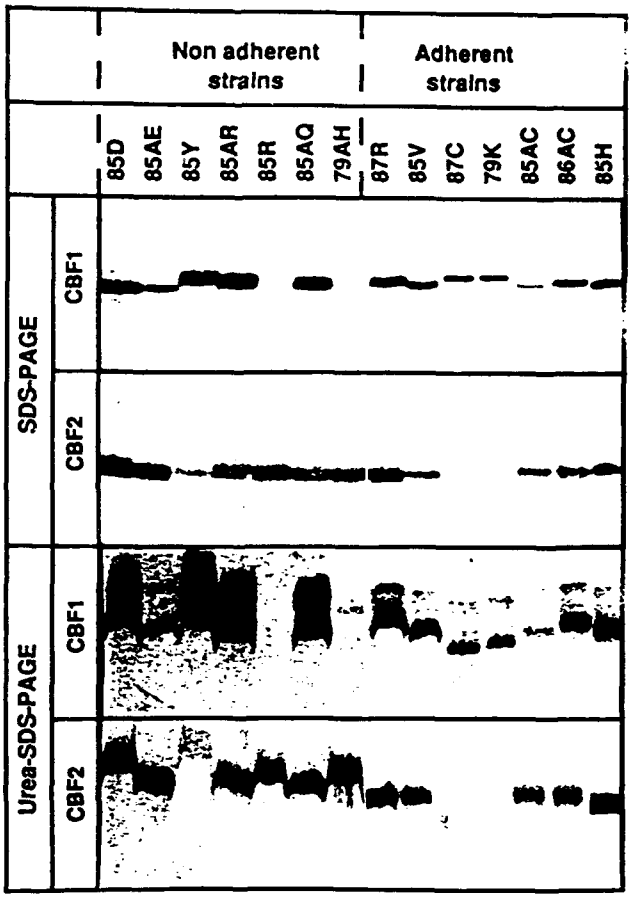


FIG. 5. Immunoblotting of whole-cell lysates from adherent and nonadherent *C. jejuni* or *C. coli* strains with anti-CBF1 and anti-CBF2. Equivalent pellets of various *Campylobacter* cells (OD_{600} = 1.0) were separated on 11% acrylamide (upper panel) or 11% acrylamide–7 M urea (lower panel) SDS-PAGE and electrotransferred. Bands were resolved with hyperimmune rabbit serum to either CBF1 or CBF2.

while the binding ability of CBF1 was similar to that of the glycine extract from adherent strain 85H. Thus, only isolated CBF1 is endowed with the adhesive property characteristic of CBFs. In this experiment, relative adherence increased as the protein concentration of the original inoculum increased. This phenomenon likely represents aggregation of other proteins to the truly adherent ones, which then would be detected by the broadly specific antiserum. An alternative explanation may be an increase in nonspecific binding due to high protein concentrations.

In these experiments, it was possible that the purified proteins had been partially denatured and their true adherence was underestimated. Therefore, to examine the adherence of CBFs in their native state, we used the antisera to CBF1 and CBF2 as immunological probes to detect the adherent components of the glycine extract that could bind to HeLa cell membranes (Fig. 7B). When used in these native conditions, CBF1 continued to exhibit greater adherence than CBF2.

Inhibition of CBF adherence by detergents. To learn whether hydrophobic interactions play a role in the binding of CBFs to HeLa cell membranes, we used the microassay to assess the adhesion of glycine extract from adherent strain 85H or of CBF1 and CBF2 in the absence or presence of either Tween 20 (0.05%, wt/vol) or SDS (0.05%, wt/vol). The adherence of the glycine extract and CBF1 was weakly reduced by Tween 20 and markedly reduced by SDS (Fig. 8). The adherence of CBF2 was lowered by both Tween 20 and SDS. These results suggest that hydrophobic interactions participate in the adherence of the CBFs, especially CBF2, to HeLa cells.

Inhibition of *C. jejuni* adherence by antisera to CBF1 and CBF2. To determine further whether CBF1 and CBF2 from *C. jejuni* 85H mediate the adherence of whole bacteria, hyperimmune rabbit sera to these proteins were assayed for the ability to inhibit adherence of bacterial cells to viable HeLa cells. These sera were initially adjusted to the same titers as previously described (11). After incubation of bacteria with appropriate serum dilutions, the adherence of bacterial whole cells to viable HeLa cells was evaluated. Three specific rabbit sera were studied: anti-CBF1, anti-CBF2, and anti-P23 (the 23-kDa protein). P23 was used as a nonbinding control outer membrane protein. Immunogold studies using monospecific antiserum to P23 demonstrated that this protein is exposed on the surface of whole bacterial cells (data not shown). We did not use anti-FLA as a control because the assay used living bacteria, and inhibition of motility by anti-FLA could nonspecifically inhibit adherence (30). The inhibitory effect of these three sera on the adhesion of strain 85H is shown in Fig. 9. The complete (100%) adherence level was established by incubating HeLa cell monolayers with *C. jejuni* organisms treated with nonimmune rabbit serum. Antiserum to CBF1 strongly inhibited the adhesion of *C. jejuni* (nearly 80% inhibition at a dilution of 1:100). In contrast, a much lower level of inhibition was observed when antiserum to CBF2 or P23 was used.

Inhibition of *C. jejuni* adherence by purified CBFs. To determine whether the purified CBFs could competitively inhibit *C. jejuni* adhesion, HeLa cell monolayers were incubated with the proteins before the adherence assay (Fig. 10). Preincubation of the cells with BSA had little effect, but preincubation with CBF1 substantially diminished the adherence of *C. jejuni* in a dose-dependent manner. Inhibition by CBF2 was much lower.

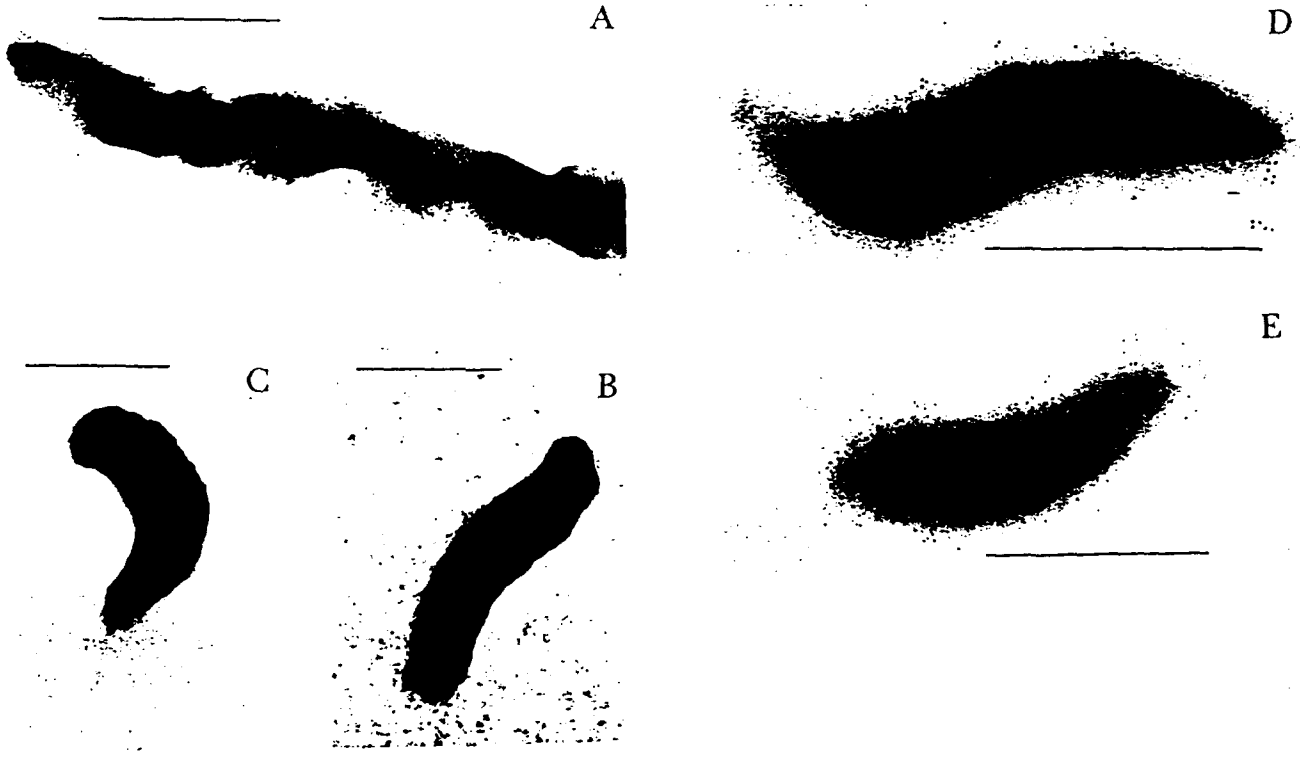


FIG. 6. Immunogold labelling of *C. jejuni* 85H with anti-CBF1 serum (A), anti-CBF2 serum (B), preimmune rabbit serum (C), anti-PEB1 serum (D), and antiserum to glycine-extracted proteins between 25 and 45 kDa (E). Bars, 1 μm.

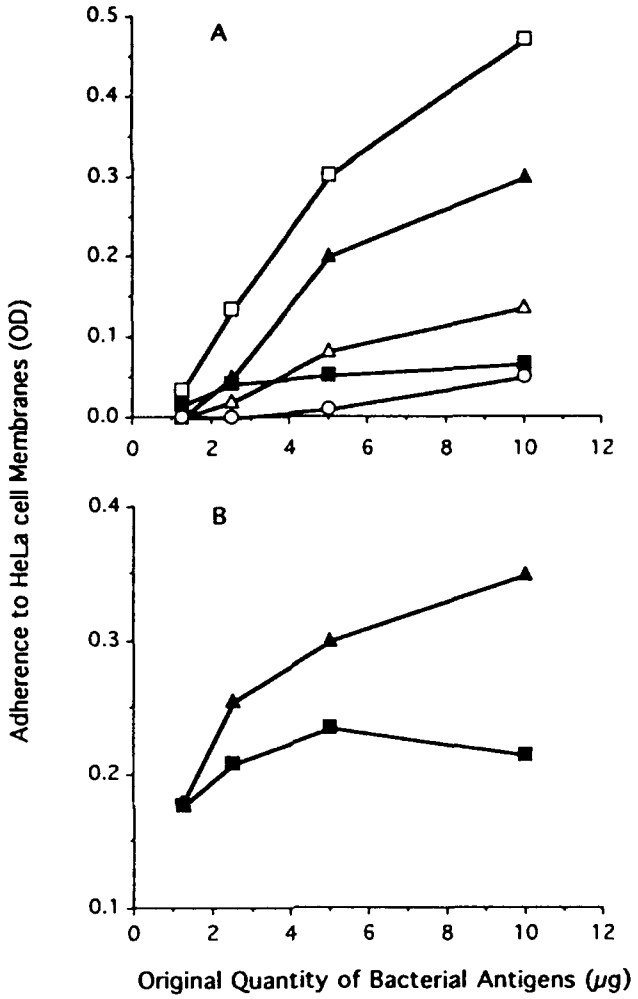


FIG. 7. Adherence of purified or native CBFs to HeLa cell membranes. (A) Adherence of purified CBF1, CBF2, and FLA from *C. jejuni* 85H. The adherence of purified CBF1 (▲), CBF2 (■), and FLA (△) was compared with that of crude glycine extracts from *C. jejuni* 85H (□) and 79AH (○) by using an adherence microassay (10). Adhering ODs were determined by using a rabbit polyclonal antiserum to *C. jejuni* whole cells (see Materials and Methods). (B) Adhering material present in glycine extract from *C. jejuni* 85H detected with antiserum to CBF1 or CBF2. The adherence microassay (10) was performed with crude glycine extract from strain 85H. After adherence to the HeLa cell membranes, the adhering components of this extract were detected by antiserum to CBF1 (▲) or CBF2 (■). The results shown are means of duplicate determinations.

DISCUSSION

A group of proteins (previously termed CBFs) including two major proteins (CBF1 and CBF2) of adherent *C. jejuni* have previously been shown to bind to eucaryotic cells (11). We obtained CBFs by preparative electrophoresis, electroelution, and electrodialysis. This method provided substantial amounts of CBF1 and CBF2 purified to homogeneity and sufficiently renatured to maintain antigenicity and adherence activity. Immunologic reactivities demonstrated that CBF1 and CBF2 are distinct proteins and represent the previously described antigens PEB1 and PEB4, respectively (27). Our electron microscopic data indicate that CBF1, but not CBF2, is surface exposed. CBF2 recovered from bacterial cells by mild solubilization conditions is probably not an integral membrane protein. Dubreuil et al. (6) studied a major *C. jejuni* protein of 30 to 31 kDa obtained by glycine extract in the same manner as CBF2. Since CBF2 is PEB4 and PEB4 is the same size and has the same amino-terminal sequence as Dubreuil's protein (27), we believe that these all represent the same protein. Dubreuil found that digestion of *Campylobacter* cells with trypsin does not remove the protein and, by using immunogold electron microscopy, that the protein is closely associated with the inner membrane (6). These findings are consistent with our electron microscopic data and with our finding that CBF2 is a nonadherent protein when isolated. CBF2 (PEB4, the Dubreuil 31-kDa protein) is

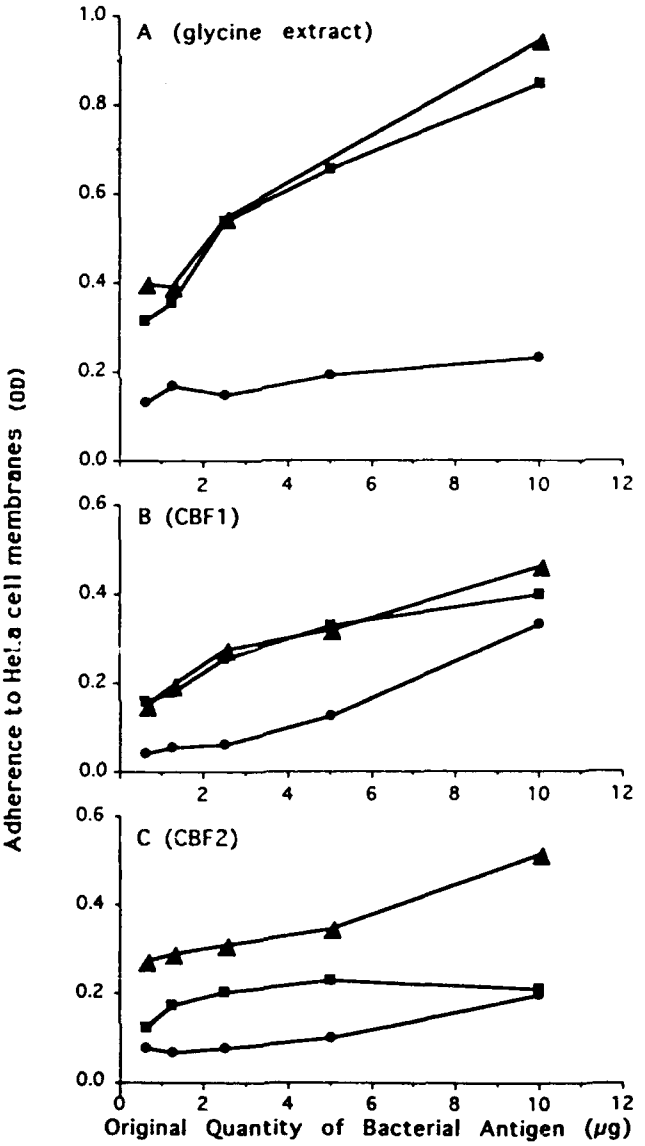


FIG. 8. Inhibition of adherence of glycine extract (A), CBF1 (B), or CBF2 (C) to HeLa cell membranes by Tween 20 or SDS. The adherence microassay was performed with either PBS (pH 7.4) alone (▲), PBS with 0.05% Tween 20 (■), or PBS with 0.05% SDS (●) in the wash solutions. The results shown are means of duplicate determinations.

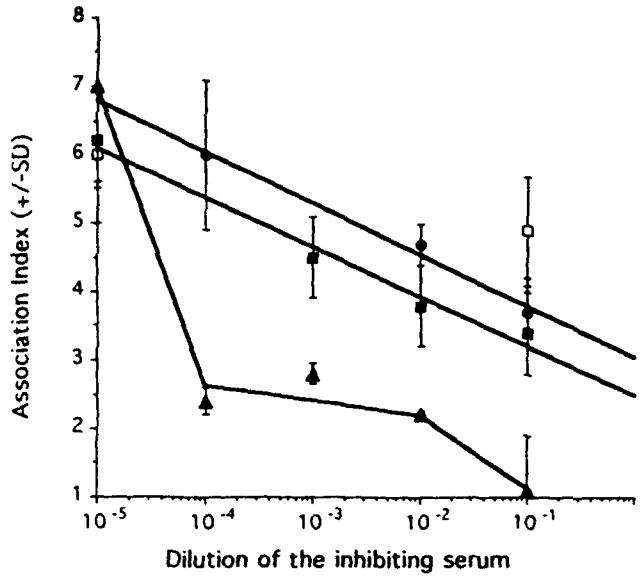


FIG. 9. Inhibition of adherence of *C. jejuni* 85H to HeLa cells by three hyperimmune rabbit sera. Adhesion of viable bacterial cells to cultivated HeLa cells was performed by using bacteria previously treated by nonimmune rabbit serum (□), anti-CBF1 serum (▲), anti-CBF2 serum (■), or anti-P23 serum (●). Each point represents the mean ± the standard deviation of three to five separate determinations. The AI represents the mean number of adhering bacteria per cell.

probably not surface exposed. These findings also are consistent with a recent study on *C. jejuni* proteins isolated by glycine extraction (27) that showed that PEB1 (CBF1) is commonly recognized by convalescent-phase sera from either *C. jejuni*- or *C. coli*-infected patients but that PEB4 (CBF2) is not.

Although strain 85R exhibits an undetectable level of

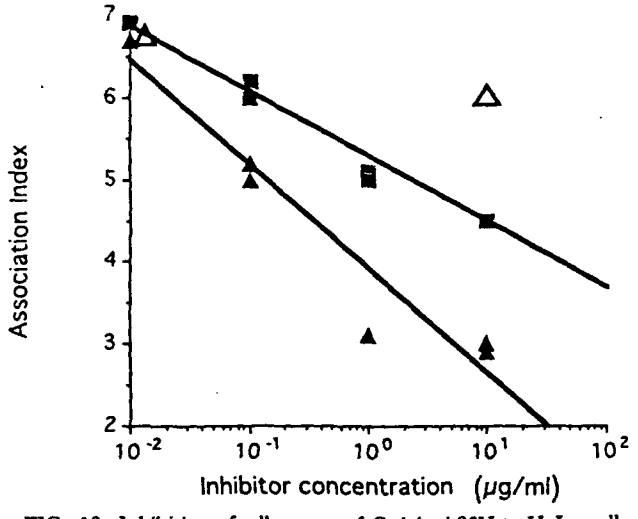


FIG. 10. Inhibition of adherence of *C. jejuni* 85H to HeLa cells by CBFs. Before the adhesion assay, HeLa cell monolayers were incubated with CBF1 (▲), CBF2 (■), or BSA (△) at various concentrations. After washing, the HeLa cells were used to determine the AI of strain 85H under standard assay conditions (13). The results shown are means of two separate determinations.

CBF1, all of the other strains tested possess CBF1 and CBF2, but in varied amounts; whether or not strain 85R possesses the gene that encodes CBF1 is under investigation. As has been previously reported (5), we could not distinguish between adherent and nonadherent strains on the basis of their SDS-PAGE protein profiles (11) but the present analysis by urea-SDS-PAGE indicates different profiles for adherent and nonadherent strains. The urea-SDS-PAGE system has been successfully developed to separate the OmpC and OmpF porins of *Escherichia coli*, which differ by less than 10 amino acids (26). By the urea-gel system, the presence of immunoreactive molecular forms which are detected in some strains, particularly by anti-CBF1, may either represent isoforms deriving from amino-terminal cleavage during their assembly or correlate with chemical modifications of the polypeptide chains. The localization of immunoreactive material migrating above the major antigen band (Fig. 5) suggests that this material represents CBFs that are chemically modified rather than degraded or cleaved isoforms that would migrate at lower molecular weights. These modifications might be either covalent acylation, such as previously described for Braun's *E. coli* lipoprotein (17), other posttranslational modifications, or strong interactions with other membrane components, such as lipopolysaccharide as described for porin-lipopolysaccharide complexes in *E. coli* (16). The CBF1 cross-reacting materials represent a more heterogeneous pattern than that for CBF2, in which only few products were immunodetected, suggesting differences between CBF1 and CBF2 regarding molecular conformation or interactions with other cell wall components.

As measured by an acellular microassay (10) and confirmed by a Scatchard analysis of data from Fig. 7A (data not shown), purified CBF1 binds better to HeLa cell membranes than does CBF2 or FLA. When obtained by the Sarkosyl method, which is known to separate outer from inner membrane proteins (15), the two proteins appear to be able to bind together to eucaryotic cells (11). However, in the present study, when these proteins were isolated from one another and from their original structure, the results obtained suggested that only CBF1 bears a binding site and CBF2 has lost its adherence activity. There are several possible explanations for the loss of this function. The simplest one would be that purification of this protein led to its denaturation. However, it is unlikely that the same purification method led to the denaturation of CBF2 but not of CBF1. It is also improbable that denaturation could lead to the acquisition of such a biological property by CBF1. Furthermore, similar results were observed in experiments in which we used the crude glycine extract as a bacterial ligand (Fig. 7B), this extract contains the proteins in their native conformation and retaining their biological properties. Another possibility is that CBF2 is not a binding protein but under native condition coadheres with CBF1 because of noncovalent linkages. The effects of detergents on CBF binding suggest that hydrophobic interactions are involved in CBF adherence, especially in CBF2 adherence. The exact mechanisms by which these proteins are involved in the attachment of bacteria to cells are not known. Purified CBF1 competitively reduces the adherence of whole bacterial cells better than does CBF2 or BSA as a negative control. We found that polyclonal antiserum to CBF1 inhibits *C. jejuni* adhesion to a much greater extent than does anti-CBF2 serum or antiserum to P23, a nonadhering outer membrane protein. In a previous work, we demonstrated that antiserum to P92 (another outer membrane protein) did not inhibit the adherence of whole bacterial cells (11). Therefore, CBF1

appears to be especially required for adhesion of *C. jejuni* to HeLa cells, whereas CBF2 does not seem to be essential for this property.

Whether CBF1 is involved in in vivo adherence of *C. jejuni* to the intestinal wall was not the goal of this study but was an underlying question. Favoring this hypothesis is the finding that infected patients exhibit a strong antibody response to this protein (27). However, the same is true for FLA, which also has been suspected to be an adhesin (30). Anti-CBF1 rabbit serum inhibits *C. jejuni* whole cell adherence to HeLa cells, as does competitively purified CBF1, but HeLa cells are different from the enteric mucosa. Finally, only in vivo experiments using an animal model would lead to convincing data addressing the role of CBF1 as an adhesin.

We conclude that CBF1 is a surface-exposed and immunogenic protein of *C. jejuni* that specifically binds to eucaryotic cell membranes and could potentially play a role in adherence to intestinal cells; however, further studies are necessary. CBF2 is probably linked to CBF1 in the cell wall structure of *C. jejuni* but is not surface exposed and does not bind to cellular membranes. Differences in isoforms of CBF1 could help explain the adherence difference among *C. jejuni* strains.

ACKNOWLEDGMENTS

This work was partially supported by the Philippe Fondation, the Beecham and IPSEN Institutes, the U.S. Army Medical Research and Development Command, and the Thrasher Research Fund.

The transmission electron microscopy studies were performed by B. Fernandez and N. Quellard in the Pathology Laboratory of CHU of Poitiers under the direction of P. Babin. We thank Michel Véron for helpful discussions.

REFERENCES

1. **Blaser, M. J., J. A. Hopkins, R. M. Berka, M. L. Vasil, and W. L. L. Wang.** 1983. Identification and characterization of *Campylobacter jejuni* outer membrane proteins. Infect. Immun. **42**:276–284.
2. **Blaser, M. J., J. A. Hopkins, and M. L. Vasil.** 1984. *Campylobacter jejuni* outer membrane proteins are antigenic for humans. Infect. Immun. **43**:986–993.
3. **Burnette, W. N.** 1984. Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
4. **de Melo, M. A., G. Gabbiani, and J.-C. Pechère.** 1989. Cellular events and intracellular survival of *Campylobacter jejuni* during infection of HEp-2 cells. Infect. Immun. **57**:2214–2222.
5. **de Melo, M. A., and J.-C. Pechère.** 1990. Identification of *Campylobacter jejuni* surface proteins that bind to eucaryotic cells in vitro. Infect. Immun. **58**:1749–1756.
6. **Dubreuil, J. D., M. Kostrzynska, S. M. Logan, L. A. Harris, J. W. Austin, and T. J. Trust.** 1990. Purification, characterization, and localization of a protein antigen shared by thermophilic campylobacters. J. Clin. Microbiol. **28**:1321–1328.
7. **Dunn, B. E., M. J. Blaser, and E. L. Snyder.** 1987. Two-dimensional gel electrophoresis and immunoblotting of *Campylobacter* outer membrane proteins. Infect. Immun. **55**:1564–1572.
8. **Dzandu, J. K., M. E. Deh, D. L. Barratt, and G. E. Wise.** 1984. Detection of erythrocyte membrane proteins, sialoglycoproteins and lipids in the same polyacrylamide gel using a double staining technique. Proc. Natl. Acad. Sci. USA **81**:1733–1734.
9. **Evans, D. G., D. J. Evans, Jr., and D. Y. Graham.** 1989. Receptor-mediated adherence of *Campylobacter pylori* to mouse Y-1 adrenal cell monolayers. Infect. Immun. **57**:2272–2278.
10. **Fauchère, J. L., and M. J. Blaser.** 1990. Adherence of *Helicobacter pylori* cells and superficial components to HeLa cell membranes. Microb. Pathog. **9**:427–439.
11. **Fauchère, J. L., M. Kervella, A. Rosenau, K. Mohanna, and M. Véron.** 1989. Adhesion to HeLa cells of Campylobacter jejuni and C. coli outer membrane components. Res. Microbiol. **140**:379–392.
12. **Fauchère, J. L., and A. Rosenau.** 1991. *Campylobacter* et *Helicobacter* en pathologie digestive humaine. Med. Sci. **7**:138–152.
13. **Fauchère, J. L., A. Rosenau, M. Véron, E. N. Moyen, S. Richard, and A. Pfister.** 1986. Association with HeLa cells of *Campylobacter jejuni* and *C. coli* isolated from human feces. Infect. Immun. **54**:283–287.
14. **Fauchère, J. L., M. Véron, A. Lellouch-Tubiana, and A. Pfister.** 1985. Experimental infection of gnotobiotic mice with Campylobacter jejuni: colonization of intestine and spread to lymphoid and reticulo-endothelial organs. J. Med. Microbiol. **20**:215–225.
15. **Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart.** 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. **115**:717–722.
16. **Hancke, K., and V. Braun.** 1973. Covalent binding of lipid to protein diglyceride and amide-linked fatty acid at the N-terminal end of the murein lipoprotein of the *E. coli* outer membrane. Eur. J. Biochem. **34**:284–296.
17. **Holzenburg, A., A. Engel, R. Kessler, H. J. Manz, A. Lustig, and U. Aebi.** 1989. Rapid isolation of OmpF porin-LPS complexes suitable for structure-function studies. Biochemistry **28**:4187–4193.
18. **Kervella, M.** 1990. Identification des protéines bactériennes impliquées dans la fixation des campylobacter aux cellules eucaryotes. Ph.D. thesis. Université de Paris XI.
19. **Konkel, M. E., F. Bababkani, and L. A. Joens.** 1990. Invasion-related antigens of *Campylobacter jejuni*. J. Infect. Dis. **162**: 888–895.
20. **Konkel, M. E., and L. A. Joens.** 1989. Adhesion to and invasion of HEp-2 cells by *Campylobacter* spp. Infect. Immun. **57**:2984–2990.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
22. **Logan, S. M., and T. J. Trust.** 1982. Outer membrane characteristics of *Campylobacter jejuni*. Infect. Immun. **38**:898–906.
23. **Logan, S. M., and T. J. Trust.** 1983. Molecular identification of surface protein antigens of *Campylobacter jejuni*. Infect. Immun. **42**:675–682.
24. **McCoy, C. E., D. Doyle, K. Burda, L. B. Corbeil, and A. J. Winter.** 1975. Superficial antigens of *Campylobacter* (*Vibrio*) *fetus*: characterization of an antiphagocytic component. Infect. Immun. **11**:517–525.
25. **Newell, D. G., H. McBride, and J. M. Dolby.** 1985. Investigations on the role of flagella in the colonization of infant mice with *Campylobacter jejuni* and attachment of *Campylobacter jejuni* to human epithelial cell lines. J. Hyg. **95**:217–227.
26. **Pages, C., P. Prince, and J. M. Pages.** 1987. Immunological comparison of major outer membrane proteins from different strains of *Escherichia coli*. Ann. Inst. Pasteur/Microbiol. (Paris) **138**:393–406.
27. **Pei, Z., R. T. Ellison, and M. Blaser.** 1991. Identification, purification and characterization of major antigenic proteins of *Campylobacter jejuni*. J. Biol. Chem. **226**:16363–16369.
28. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Pirovenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150**:76–85.
29. **Tessmer, U., and R. Dermick.** 1989. Preparative separation of poliovirus structural polypeptides by SDS-PAGE, copper staining and electroelution and induction of monospecific antisera. Electrophoresis **10**:277–279.
30. **Walker, R. I., M. B. Caldwell, E. C. Lee, P. Guerry, T. J. Trust, and G. M. Ruiz-Palacios.** 1986. Pathophysiology of *Campylobacter* enteritis. Microbiol. Rev. **50**:81–94.
31. **Walker, R. I., E. A. Schmauder-Chock, J. L. Parker, and D. Burr.** 1988. Selective association and transport of *Campylobacter jejuni* through M cells of rabbit Peyer's patches. Can. J. Microbiol. **34**:1143–1147.